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The following major discoveries have resulted from contract DAAL03-86-K-0130:

1] We have established procedures for the dissociation and reassembly of ciliary membranes from olfactory epithelium under conditions that allow survival of the olfactory adenylate cyclase. These studies represent a first step toward the molecular dissection and functional reconstitution of the chemosensory membrane.

2] We have discovered that the olfactory adenylate cyclase is regulated by calcium/calmodulin and characterized the effects of calcium/calmodulin on modulation of this enzyme. These studies underscore the importance of crosstalk between second messenger systems in olfactory transduction and indicate a central role for calcium in the chemoreceptive process.

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3] We have characterized proteins that are substrates for cyclic AMP-dependent protein kinase in ciliary and axonal membranes of olfactory receptor cells. In addition, we have identified a protein of which the phosphorylation proceeds independent of cyclic nucleotides and is inhibited by low concentrations of calcium.

4] We have established a library of monoclonal antibodies against olfactory cilia and identified several epitopes that are unique to chemosensory cilia and absent from olfactory nerve membranes, respiratory cilia and membranes from a host of other tissues. One of these proteins forms membrane-associated oligomers linked together by intermolecular disulfide bonds. All, but one, of these olfactory cilia-specific proteins are identified by antibodies that react with unique carbohydrate moieties. This group of novel olfactory specific proteins is likely to contain odorant receptors or olfactory cilia-specific transduction proteins.

Future studies under contract DAAL03-89-K-0178 will focus on the role of calcium in olfactory transduction and on the molecular cloning and characterization of olfactory cilia specific proteins identified by our monoclonal antibodies.

PRIMARY EVENTS IN OLFACTORY RECEPTION

FINAL REPORT

ROBERT R. H. ANHOLT, PH. D.

OCTOBER 5, 1989

U. S. ARMY RESEARCH OFFICE

DAAL03-86-K-0130

DUKE UNIVERSITY MEDICAL CENTER

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I am pleased to report that contract DAAL03-86-K-0130 has been succesfully completed and has, in fact, yielded more information than originally anticipated. The following major discoveries have resulted from this contract:

1) We have established procedures for the dissociation and reassembly of ciliary membranes from olfactory epithelium under conditions that allow survival of the olfactory adenylate cyclase. These studies represent a first step toward the molecular dissection and functional reconstitution of the chemosensory membrane.

2) We have discovered that the olfactory adenylate cyclase is regulated by calcium/calmodulin and characterized the effects of calcium/calmodulin on modulation of this enzyme. These studies underscore the importance of crosstalk between second messenger systems in olfactory transduction and indicate a central role for calcium in the chemoreceptive process.

3) We have characterized proteins that are substrates for cyclic AMP dependent protein kinase in ciliary and axonal membranes of olfactory receptor cells. In addition, we have identified a protein of which the phosphorylation proceeds independent of cyclic nucleotides and is inhibited by low concentrations of calcium.

4) We have established a library of monoclonal antibodies against olfactory cilia and identified several epitopes that are unique to chemosensory cilia and absent from olfactory nerve membranes, respiratory cilia and membranes from a host of other tissues. One of these proteins forms membrane-associated oligomers linked together by intermolecular disulfide bonds. All, but one, of these olfactory cilia-specific proteins are identified by antibodies that react with unique carbohydrate moieties. This group of novel olfactory specific proteins is likely to contain odorant receptors or olfactory cilia-specific transduction proteins.

Future studies under contract DAAL03-89-K-0178 will focus on the role of calcium in olfactory transduction and on the molecular cloning and characterization of olfactory cilia specific proteins identified by our monoclonal antibodies.

The major findings of work performed under contract DAAL03-86-K-0130 are summarized in greater detail in the following paragraphs.

1) Olfactory transduction

I. Adenylate cyclase in dendritic cilia and axons of olfactory receptor cells.

Olfactory cilia contain a high level of adenylate cyclase activity, which can be stimulated in a GTP-dependent manner by micromolar to millimolar concentrations of some, but not all, odorants. Studies of homologous series of odorants showed their ability to activate the olfactory adenylate cyclase to be proportional to their octanol/water partition coefficient. This, together with the observation that odorants stimulate the adenylate cyclase of frog melanophores in a similar manner, suggests that partitioning of odorants in the membrane rather than specific odorant receptor proteins may mediate the observed stimulation of the olfactory adenylate cyclase.

We have developed a preparation of olfactory nerve membranes to assess whether adenylate cyclase is present only in the dendritic cilia or whether it also occurs in other regions of the olfactory receptor cell. In the bullfrog, clean stretches of olfactory nerve up to 1 cm in length can be dissected and olfactory nerve membranes that contain axonal membranes of olfactory receptor cells can be prepared.

We found that olfactory nerve membranes contain substantial adenylate cyclase activity, comparable to levels encountered in brain membranes. The specific activity of the axonal enzyme is, however, approximately 20-fold lower than that of the ciliary enzyme. The axonal enzyme is not activated by odorants and requires app. 20-fold higher concentrations of the non-hydrolyzable GTP analogue, GTPγS for activation via its regulatory GTP-binding protein. Forskolin stimulates the axonal and the ciliary enzyme with the same EC_{50} (1-3 μM).

Immunochemical studies with antisera against the α subunits of GTP-binding proteins show that olfactory nerve membranes like dendritic cilia contain G_s and G_o, although G_o is more prominent in the axonal membrane preparation than in the cilia. Studies from other laboratories suggest that axonal membranes contain conventional G_s whereas the cilia contain an olfactory specific variant of G_s, designated G_{olf}. Failure of the axonal enzyme to respond to odorants and its lower sensitivity to GTPγS may be related to its lower concentration in the membrane which lowers the probability for collisional interactions between the adenylate cyclase and the activated G-protein by lateral diffusion and may also render the system less sensitive to membrane perturbations resulting from partitioning of odorants. It can, however, not be excluded that the ciliary enzyme is stimulated via odorant receptor proteins that are not present on the axons.

The role of the olfactory adenylate cyclase is not yet clear. We have tested many neuroactive agents, including histamine, adenosine, isoproterenol, prostaglandins, serotonin, acetylcholine, neuropeptide Y, taurine and carnosine, as well as pituitary extract and commercially available media growth supplements. None of these substances affected either the ciliary or the axonal enzyme.

II. Functional reconstitution of the olfactory adenylate cyclase

To facilitate further biochemical investigations of the olfactory adenylate cyclase, we have developed procedures that allow the functional reconstitution of this enzyme in liposomes. We found that during solubilization with Lubrol PX, the enzyme can be stabilized by supplementary soybean lipid, forskolin and sodium fluoride. Subsequent removal of the detergent by adsorption onto Biobeads SM2 followed by dialysis to remove the added forskolin and fluoride results in the formation of proteoliposomes that display forskolin- and GTPγS-sensitive adenylate cyclase activity. Sucrose gradient centrifugation of liposomes formed in the presence of fluorescent phosphatidylcholine demonstrated association between the olfactory adenylate cyclase and the exogenous lipid.

Forskolin stimulates the reconstituted enzyme with the same potency (EC_{50} = 1-2 μM) as the enzyme in the native membrane. However, GTPγS is app. 350-fold more potent in native membranes (EC_{50} = 4.0±0.5 nM) than in reconstituted membranes (EC_{50} = 1.4±0.3 μM). Thus,

the forskolin-sensitive catalytic moiety of the adenylate cyclase remains intact during the solubilization and reconstitution procedures and it retains its ability to interact with its regulatory GTP-binding protein. However, the reconstituted ciliary enzyme is no longer sensitive to odorants. Thus, the reconstituted ciliary adenylate cyclase resembles the axonal enzyme in its unresponsiveness to odorants and its lowered sensitivity to GTPγS. This may be due to the lower concentration of the enzyme and its associated G-protein in the reconstituted membrane, which may reduce the efficacy of their interactions, as elaborated above. The possibility that putative odorant receptors are denatured during the reconstitution process can, however, not be excluded. The procedures established for the incorporation of the olfactory adenylate cyclase in liposomes represent a first step toward the molecular dissection of the olfactory membrane.

III. Modulation of the olfactory adenylate cyclase by calcium/calmodulin

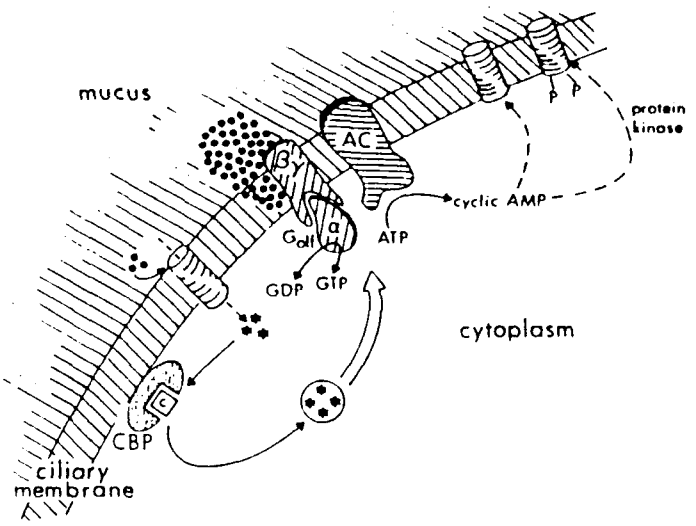
Calcium at concentrations above 10 μM inhibits the olfactory adenylate cyclase. However, at lower concentrations calcium activates the enzyme via calmodulin. Endogenous calmodulin can readily be demonstrated in olfactory cilia preparations and amounts to 1.35±0.36 μg/mg protein. Calmodulin potently stimulates the olfactory adenylate cyclase. This stimulation is half-maximal at 5.6±0.9 μM calmodulin and saturates at about 30 μM. Maximal activation of the enzyme by calmodulin is 3-6 times greater than that observed with odorants and is dependent on calcium. Estimates of free calcium concentrations in EGTA-buffered solutions show that a rise in calcium concentration between app. 0.1 and 2 μM leads to calcium/calmodulin mediated activation of the olfactory adenylate cyclase.

The mechanism of activation of the olfactory adenylate cyclase by calcium/calmodulin involves a GTP-dependent and a GTP-independent component. Stimulation observed by GTP and calmodulin together is synergistic and results in approximately 27% greater adenylate cyclase activity than the activity predicted from summation of the GTP mediated activation and stimulation by calmodulin measured separately. The GTP-dependent component of calcium/calmodulin induced adenylate cyclase activation is also inhibited by GDPβS.

In contrast to the synergism observed between GTP and calmodulin, activation by odorants and calmodulin is additive both at submaximal and at saturating concentrations of calmodulin. Thus, odorants at high micromolar concentrations and calmodulin activate the olfactory adenylate cyclase via independent mechanisms.

Previous models of olfactory transduction have focused on activation of adenylate cyclase by odorants without considering a role for calcium. Our demonstration that calcium at physiological concentrations activates this enzyme through calmodulin via a GTP-dependent and a GTP-independent mechanism establishes a role for calcium in olfaction and underscores the importance of crosstalk between second messenger systems in olfactory transduction. Our data suggest that odorants at physiological concentrations may cause an influx of calcium which then leads to a rise in intracellular cyclic AMP levels via the formation of a calcium/calmodulin complex. Only at high micromolar concentrations of odorants would the calcium pathway be bypassed by direct activation of the olfactory adenylate cyclase. These proposed transduction mechanisms are schematically depicted in the diagram below, in which

CBP designates calmodulin binding proteins, *c* designates calmodulin, *AC* designates adenylate cyclase and the stars represent calcium ions:



Olfactory receptor neurons possess a high membrane impedance. These cells are electrotonically compact and only picoamperes of injected current are sufficient to elicit action potentials. Consequently, tight regulation of the membrane potential at rest and during activation by odorants is essential for these highly sensitive cells. The second messenger cascade which we propose would provide at the same time signal amplification and multiple points of control over the cellular response to odorants.

IV. Phosphorylation of proteins in olfactory cilia

To gain insight into the function of cyclic AMP in olfaction it is important to identify proteins that are substrates for cyclic AMP-dependent protein kinase. Incubation of olfactory cilia with cyclic AMP, ^{32}P-γATP and commercially available cyclic AMP-dependent protein kinase from bovine heart results in the phosphorylation of a large number of proteins as assessed by polyacrylamide gel electrophoresis and autoradiography. The specificity of these phosphorylation reactions can be assessed by conducting the reactions in the presence of protein kinase inhibitor or in the absence of cyclic AMP. Under these conditions phosphorylation is reduced to a low basal level of endogenous activity. Phosphorylation is reversible by treatment of phosphorylated cilia with alkaline phosphatase. Comparing phosphorylation of proteins of olfactory cilia with those of respiratory cilia indicates that cytoskeletal proteins, especially tubulin, are among the most effective substrates for cyclic AMP-dependent protein kinase. Comparing phosphorylation patterns of dendritic cilia with olfactory nerve membranes shows that the patterns of phosphorylation in the cilia and axons of olfactory receptor cells are virtually identical and that under the conditions used it is difficult to detect phosphoproteins that are unique to the cilia.

Phosphorylation of olfactory cilia by incubation with radioactive ATP has, however, two pitfalls: 1) It is difficult to assess whether the phosphorylated proteins are relevant substrates for cyclic AMP-dependent protein kinase under physiological conditions; and, 2) Proteins that are already phosphorylated on a cyclic AMP-dependent phosphorylation site and, hence, are physiologically relevant substrates may remain undetected.

To circumvent these problems we have, in collaboration with Drs. Guy Salvesen and Jan Enghild (Dept. Pathology, Duke Univ. Med. Ctr.), synthesized the octapeptide *Gly-Leu-Arg-Arg-Ser-Ser-Leu-Gly*, which represents a "generic" phosphorylation site for cyclic AMP dependent phosphorylation. Indeed, this peptide is readily phosphorylated by cyclic AMP-dependent protein kinase in the presence of ATP and cyclic AMP. We have developed conditions under which 80-100% of the peptide can be enzymatically phosphorylated. Moreover, sequence analysis of the phosphorylated peptide demonstrates that the phosphate group is selectively transferred onto the second serine. The phosphorylated product, *Gly-Leu-Arg-Arg-Ser-Ser(**P**)-Leu-Gly*, is readily separable by HPLC from the non-phosphorylated peptide and can be conjugated to bovine serum albumin or ovalbumin using gluteraldehyde as the crosslinker. We have raised both polyclonal antisera in rabbits and monoclonal antibodies in mice against these conjugates. Using ELISA assays and preimmune sera as controls we have shown that the antisera react specifically with the phosphorylated peptide and not with the non-phosphorylated peptide or individual phosphorylated amino acids, such as phosphoserine and phosphotyrosine. We will use these antisera to identify ciliary proteins that contain a phosphorylated consensus site for cyclic AMP-dependent phosphorylation. Phosphoproteins identified by these antibodies as relevant *in vivo* substrates for cyclic AMP-dependent protein kinase, that are enriched in or unique to olfactory cilia by comparison with olfactory nerve membranes, are likely to play an important role in olfaction and the identification and characterization of these proteins will be continued under contract DAAL03-89-K-0178.

In the course of our studies on protein phosphorylation in olfactory cilia we discovered a 48 kDa polypeptide which becomes rapidly phosphorylated during incubation of olfactory cilia with 10-20 nM 32P-γATP. Phosphorylation of this polypeptide occurs in the absence of added cyclic nucleotides and is inhibited by nanomolar concentrations of calcium. We refer to this calcium-regulated phosphoprotein as *CARPP48*.

CARPP48 is not detected in non-chemosensory cilia from respiratory epithelium. It is, however, prominent in brain membranes and is also observed in olfactory nerve membranes. When intensities of autoradiographic bands are compared it is clear that olfactory cilia contain much higher concentrations of CARPP48 than olfactory nerve membranes. CARPP48 appears to be a peripheral membrane protein, since it is recovered in the detergent-poor phase after extraction with Triton X-114 and does not bind either concanavalin A or wheat germ agglutinin.

When phosphorylation is allowed to proceed in the absence of calcium, subsequent addition of calcium does not result in dephosphorylation of CARPP48, indicating that calcium does not act by stimulating a phosphatase, but rather by inhibiting a kinase. It is not clear which endogenous kinase phosphorylates CARPP48. Olfactory cilia contain protein kinase C and it is possible that a catalytically active proteolytic fragment of this enzyme with altered regulatory properties performs the phosphorylation of CARPP48.

Olfactory cilia contain a calcium-dependent ATPase which is activated at app. 1 nM calcium, half-maximally active at about 15 nM calcium and fully active at calcium concentrations above 100 nM (V_{max} in olfactory cilia = 11.0±1.6 nmol ATP hydrolyzed/min/mg protein; in olfactory nerve membranes: 5.4±0.2 nmol ATP hydrolyzed/min/mg protein). At calcium concentrations above 100 nM, phosphorylation of CARPP48 is fully suppressed, but phosphorylation of polypeptides of 92 kDa and 97 kDa, possibly representing autophosphorylated forms of the calcium-dependent ATPase, becomes evident.

Although the function of CARPP48 is not clear, its enrichment in olfactory cilia and inhibition of its phosphorylation by physiological concentrations of intracellular calcium suggest a role for this protein in olfactory transduction. We will continue to study CARPP48 under contract DAAL03-89-K-0178.

2) *Identification of olfactory cilia specific proteins by monoclonal antibodies*

To identify proteins unique to olfactory cilia we have immunized mice with frog olfactory cilia and raised a library of monoclonal antibodies against ciliary proteins. Monoclonal antibody producing cell lines were identified via a solid phase ELISA assay. Initially, we identified a total of 36 stable monoclonal antibody producing hybridomas, which have been subcloned twice. Nineteen of these antibodies react with proteins immobilized on a nitrocellulose membrane after polyacrylamide gel electrophoresis in SDS. The rest of the antibodies are either low affinity antibodies or directed against conformationally dependent determinants.

Thus far, we have focused our attention on those antibodies that react with nitrocellulose-immobilized proteins. Immunoblots prepared with these antibodies reveal three categories of staining patterns: 1) antibodies that visualize one distinct band; 2) antibodies that visualize multiple distinct bands; and, 3) antibodies that stain one or more diffuse bands. Antibodies in the second group may either recognize proteins with homologous sequences or proteolytic fragments of one distinct membrane protein. The preponderance of this staining pattern leads us to favor the latter possibility, illustrating the high sensitivity of ciliary proteins to proteolysis during isolation. Including mixtures of various protease inhibitors during the detachment and isolation of olfactory cilia does not alter the staining patterns significantly, which suggests that proteolysis occurs rapidly after decapitation of the frog and during removal of the olfactory epithelia. The third group of antibodies is most likely directed against carbohydrate moieties found on several glycoproteins.

To investigate whether any of our monoclonal antibodies recognize proteins that are unique to olfactory cilia, we tested their reactivity with respiratory cilia and olfactory nerve membranes. Several antibodies were identified that exhibited specificity for olfactory cilia:

1) mAb 8 visualizes a 59 kDa glycoprotein. The antigenic determinant is removed by treatment of olfactory cilia with endoglycosidase H, indicating that mAb 8 reacts with a unique carbohydrate site. Comparing the reactivity of mAb8 when ciliary extracts are prepared under reducing and non-reducing conditions followed by sucrose gradient centrifugation and polyacrylamide gel electrophoresis shows that the protein recognized by mAb 8 occurs as

membrane-associated oligomers linked together by intermolecular disulfide bonds. It is of interest to note that this protein contains structural features that could account for odorant activated ion channels that have been reported to open in a cooperative manner and to display multiple equidistant conductance levels and for the previously reported sensitivity of olfactory responses to lectins and alkylating or reducing agents.

2) mAb 26 visualizes two major diffuse molecular weight regions in olfactory cilia at 56-68 kDa and 108-142 kDa. Neither of these bands are observed in respiratory cilia. Olfactory nerve membranes display the lower, but not the higher molecular weight region. Treatment of olfactory cilia with endoglycosidase H results in the progressive disappearance of first the higher and subsequently the lower molecular weight region and the appearance of a new diffuse band at 34-43 kDa. Immunization of mice with cilia pretreated with endoglycosidase H has resulted in the generation of two mAbs unique to olfactory cilia, mAb 61 and mAb 82, that resemble mAb 26 and most likely recognize the same epitope. All of these antibodies are of very high affinity. The frequency with which antibodies against this epitope occur and their high affinity suggest that this epitope unique to olfactory cilia is highly immunogenic.

3) mAb 34 is a low affinity antibody that visualizes an 87 kDa protein that is unique to olfactory cilia and is not affected by endoglycosidase H treatment.

4) mAbs 42 and 45 appear identical in their staining patterns, visualizing several bands in two diffuse staining regions of 60-66 kDa and 120-140 kDa. They do not stain proteins of respiratory cilia. In olfactory nerve membranes they stain one band at 260 kDa. These mAbs recognize carbohydrate epitopes, since immunoreactivity is abolished after pretreatment of olfactory cilia with endoglycosidase H.

5) mAb 43 stains a diffuse high molecular weight (>200 kDa) region, which is not observed in respiratory cilia and olfactory nerve membranes. Its immunoreactivity is also abolished by endoglycosidase H treatment of olfactory cilia.

None of these antibodies, described above, react with proteins in membranes prepared from brain, heart, lung, liver or kidney. In addition, they appear not to crossreact with rat olfactory tissue.

These olfactory cilia specific proteins identified by our monoclonal antibodies are likely to represent components involved in odorant recognition or olfactory transduction. The continuation of this project under contract DAAL03-K-89-0178 seeks to characterize several of these proteins using recombinant DNA technology. Sequencing of cDNAs encoding olfactory cilia specific proteins will allow us to deduce their amino acid sequences and analysis of these sequences will give information about their possible functions. These studies will greatly increase our knowledge of specific proteins of the olfactory membrane that play key roles in odorant recognition and olfactory transduction.